IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re: Yuehua Li, et al. Confirmation No.: 8515
Serial No.: 09/914,020 Group Art Unit: 1635
Filed: December 31, 2001 Examiner: Janet L. Epps-Ford
For: METHODS AND COMPOSITIONS FOR ALTERING MUCOUS SECRETION

Appendix A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket No: 5051-4511P

In re patent application of

Yuehua LI *et al.*

Group Art Unit: 1635

Serial No. 09/914,020

Examiner: Janet L. Epps-Ford

Filed: December 31, 2001

For: METHODS AND COMPOSITIONS FOR ALTERING MUCUS SECRETION

DECLARATION UNDER 37 C.F.R. §1.132

I, Indu Parikh, declare that:

1. I hold the position of President and Chief Scientific Officer with BioMarck Pharmaceuticals. I am a biochemist and have worked in a variety of areas including protein/peptide chemistry from 1966 to the present. A copy of my Curriculum Vitae is appended hereto as Exhibit 1.

2. I have read and understood the rejections based on the alleged lack of written description and alleged lack of enablement for MARCKS derived peptides other than SEQ ID NO:1 (MANS peptide, Myristoylated N-terminal Sequence) in the above-captioned application, mailed on June 6, 2005

3. In response to these rejections, I provide the results of experiments that were performed at my request showing that other myristoylated (MA) peptides of the N-terminal sequence of the MARCKS protein inhibit mucus hypersecretion in mammalian airways. Experiments were designed to test whether these peptides inhibit hypersecretion of mucin in murine airways *in vivo*. The well-established ovalbumin-sensitized mouse model of asthma was used in these studies as described in Eum *et al.*, *Proc. Natl. Acad. Sci. USA* 92: 12290-12294, 1995 (a copy is attached as Exhibit 2). As a negative control, the RNS (Randomized N-terminal Sequence, also called BIO-124R) peptide was tested along with the other peptides. Specifically, BP2 mice (or Balb/C mice), aged 6-8 wks, were immunized subcutaneously twice at weekly intervals with 1μg of ovalbumin. After 14 days of sensitization, the animals were exposed to aerosolized ovalbumin, which causes a pronounced goblet cell hyperplasia. At the 72 hr time point, the secretagogue, methacholine (60 mM) was delivered using a Boxco system nebulizer providing a fine

aerosol for 90 seconds. Fifteen minutes prior to the secretagogue challenge, 50 μL of the test peptide, at 3 different concentrations (10 μM, 100 μM, or 140 μM), was administered to the mice by intratracheal route. The RNS peptide was tested at the highest dose level only (50 μL of 140 μM solution). The various controls used in these experiments included (a) untreated saline group, (b) OVA sensitized group in absence of any peptide, and (c) group in which RNS peptide was used as a control. Each experiment was carried out in 6 mice per group, and each set of experiments was repeated 3 times. To test strain-to-strain variations, the above experiment was repeated in Balb/C mice under similar protocol. Following the methacholine challenge, the animals were sacrificed and bronchoalveolar lavage (BAL) performed on 5 animals per group for analysis of secreted mucin by ELISA method and the resulting data normalized to mg protein.

4. The following controls and peptides of the N-terminal sequence of the MARCKS protein were tested in these experiments. The data is presented in Exhibit 3 and show the results of those myristoylated peptides tested at 100 μM concentrations.

a. Saline = untreated saline group (control);
b. Ova = OVA sensitized group in absence of any peptide (control);
c. BIO-124R = RNS peptide (control);
d. BIO-124 = MANS peptide, Myristoylated 1-24 amino acid sequence of MARCKS protein.
e. BIO-120 = Myristoylated amino acid sequence 1-20 of MARCKS protein;
f. BIO-116 = Myristoylated amino acid sequence 1-16 of the MARCKS protein;
g. BIO-112 = Myristoylated amino acid sequence 1-12 of MARCKS protein; and
h. BIO-110 = Myristoylated amino acid sequence 1-10 of the MARCKS protein.

5. The results, as presented in Exhibit 3, demonstrate that the above peptides, designated BIO-124, BIO-120, BIO-116, BIO-112, and BIO-110, show between 66 to 77 % inhibition of mucin hypersecretion in the mouse model of asthma as compared to the Ova control. These data support the enablement and written description of the present invention for fragments from the N-terminal sequence of the MARCKS protein (SEQ ID NO: 3) including MANS peptide.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

September 6, 2005
Date

Indu Parikh, Ph.D.

253576 v1/RE

Attorney Docket No.: 5051-451ip PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Serial No.: 09/914,020 Group Art Unit: 1635
Filed: December 31, 2001 Examiner: Janet L. Epps-Ford
For: METHODS AND COMPOSITIONS FOR ALTERING MUCOUS SECRETION

Exhibit 1

EXHIBIT 1

INDU PARIKH, Ph.D.

4364 South Alston Ave
Durham, NC 27713
Tel: 919-768-1335
Fax: 919-361-2290
E-Mail: iparikh@cato.com

PROFESSIONAL SUMMARY:

Over 30 years of industrial drug discovery and drug development experience in design and discovery of new products and concepts in the pharmaceutical areas including controlled drug delivery systems, endocrinology, osteoporosis, mucosal diseases of the gastrointestinal tract, mucus hypersecretory pulmonary diseases, and proteins/peptides as therapeutic agents. Productive innovator and R&D leader with record of turning good ideas into profitable business opportunities and advancement of inventions to clinical development and to marketable stage.

Strengths:

- Strongly diversified technical background with business acumen
- Practical, hands-on, goal oriented approach to problem solving
- Experience in business development
- Organizational, communication and project management skills

PROFESSIONAL EXPERIENCE:

2002- **Co-Founder, President & CSO**
BioMarck Pharmaceuticals, Ltd., Durham, North Carolina

1997-2000 **Co-Founder, Chief Scientific Officer and Vice President**
RTP Pharma Inc.
Ile des Soeurs, Verdun, QC, Canada

1990-1997 **Co-Founder and Vice President for Research & Development**
Research Triangle Pharmaceuticals Ltd.
Durham, NC 27713

1986-1990 **Director**
Division of Biochemistry and Biotechnology
Glaxo Inc.
Research Triangle Park, NC

1986-1988 **Director of Extramural Research**
Glaxo Inc.
Research Triangle Park, NC

1982-1986 **Head**
Molecular Biology Department
Burroughs Wellcome Co.
Research Triangle Park, NC

1977-82 **Associate Head**
Molecular Biology Department
Burroughs Wellcome Co.
Research Triangle Park, NC

1975-77 **Group Leader**
Molecular Biology Department
Burroughs Wellcome Co., RTP, NC.

EXHIBIT 1

1970-75 **Assistant Professor of Pharmacology & Experimental Therapeutics and Assistant Professor of Medicine**
Johns Hopkins University. School of Medicine, Baltimore, MD

1968-70 **Visiting Scientist**
NIAMD, NIH, Bethesda, Maryland (Dr. Christian B. Anfinsen)

1966-68 **Postdoctoral Fellow**
Weizmann Institute of Science, Rehovot, Israel (Prof. Ephraim Katzir)

1965-66 **Research and Teaching Associate**
University of Zurich, Zurich, Switzerland

EDUCATION: Ph.D. University of Zurich, Zurich, Switzerland (1965)

PROFESSIONAL MEMBERSHIPS:

American Society of Biochemistry and Molecular Biology
American Society of Pharmacology and Experimental Therapeutics
Control Release Society
American Assoc. of Pharmaceutical Scientists
International Society for Biorecognition Technology
National Institute of Health Alumni Association

HONORS:

Monsanto Postgraduate Fellowship, 1961-63
Geigy Postgraduate Fellowship, 1963-65
Weizmann Institute Visiting Fellowship, 1966-68
Executive Vice President and Member, Board of Directors, Alopecia Areata Research Foundation, 1983-1990; 1991-1996
Member, Governing Council, Internat. Society for Biorecognition Technology, 1984-90
Treasurer, International Society for Biorecognition Technology, 1984-89
Honorary Life Member, Society of Biological Chemistry of India, 1989-
Honorary Member, National Academy of Sciences, India, 1989-

INDUSTRIAL AFFILIATIONS:

Member of Scientific/Corporate Advisory Boards of:
Bioxy International, 1989-1999
Protein Delivery Inc., (Nobex Pharmaceuticals) 1991-1997
Biomedica, Inc., 1989-1997
Cibus Pharmaceuticals, 1992-1996
Medicago, Inc., 1999-present
Bioinfinix, Inc., 1999-2002
RTP Pharma Inc., 2000-2002
PhytoMyco Research Corp., 2002-present

EXHIBIT 1

EDITORIAL BOARDS:

Journal of Solid-Phase Biochemistry, 1975-80
Journal of Applied Biochemistry and Biotechnology, 1980-86
Pharmacology and Drug Development, 1979-86

ACADEMIC APPOINTMENT:

Adjunct Professor, Pediatric Endocrinology, Department of Medicine, University of North Carolina School of Medicine at Chapel Hill, NC, 1987-1992.

MAJOR ACHIEVEMENTS:

Research Triangle Pharmaceuticals Ltd. (RTP Pharma Inc.), 1990-2000

The Company was founded on the yet to be exploited concepts in delivery of water insoluble drugs. The RTP's proprietary drug delivery technology for water insoluble drugs, DD™, was explored and employed for oral, injectable, aerosol and topical delivery of more than 100 different drugs. Two of these drugs are currently at NDA stage. The Company was initially founded in North Carolina in 1990 and later funded by a group of Quebec based institutional investors and was relocated to Montreal in 1996. RTP Pharma was acquired by SkyePharma in 2002.

The following is a few of the highlights of my contributions to RTP Pharma.

- Co-founded the Company.
- Established a data base for water insoluble drugs, performed feasibility studies, developed clientele for contract research, expanded intellectual property base for the company's proprietary drug delivery technology.
- Wrote business plan, actively participated in fund raising activities in U.S. and Canada.
- After relocation to Canada (December 1996), helped recruiting additional staff at all levels, including four VPs (Medical, Process Dev., Business Dev., and CFO), prioritized projects, and provided overall day to day R&D direction, successful in gaining R&D contracts from five large pharmaceutical companies, and actively participated in other business development activities as needed.
- Helped select drug candidates and implementation for in-house development. Lead two of these drugs to NDA stage.
- Performed proof-of-concept key experiments for a technology based on differentiation of islet precursor cells as a means to treat Type II diabetes. This technology was then spun off as a subsidiary company, Waratah Pharmaceuticals, Inc. Waratah went public in September 2000 with Canadian Small Cap CDNX exchange. This was CDNX's top selling IPO for the year 2000.

Director, Biochemistry & Biotechnology Division, Glaxo Inc., 1986-1990

The Division was active in goal oriented discovery research in biopharmaceuticals. Collaboration with University investigators was encouraged in selected areas. The Division consisted of five departments namely, Molecular Biology, Enzymology, Protein Chemistry,

Biochemical Pharmacology, and Hybridoma Technology.

- Molecular Biology Department: An important function of this Department was to express desired proteins by recombinant technology for detail biochemical and pharmacological studies. Expression systems like E. coli, mammalian cells, and yeast were routinely used. Some of the proteins expressed included:
  ■ Lipocortin I and II ■ tissue inhibitor of metalloproteases ■ EGF ■ hSOD ■ extracellular region of CD4 and ■ a fibrinolytic enzyme ■ development of vector systems to enhance the yield of cloned proteins ■ site specific mutagenesis, ■ gene regulation studies with lipocortin I and two lymphokines.
- Enzymology Department: Major efforts in the rational drug development based on designing specific inhibitors of selected metabolic enzymes. The major activities of the department included: two fibrinolytic enzymes, metalloproteases including collagenase, and 5α-reductase, and aromatase.
- Protein Chemistry Section: The section primarily provided purification and scale-up services for proteins generated by recombinant labs.
- Biochemical Pharmacology: This section investigated primarily the mucosal diseases of GI tract including gastric ulcer, atrophic gastritis, IBS, etc.: helped develop highly sensitive quantitative digital video endoscopy technology. In addition, developed a rat model for osteoporosis in collaboration with a local university.
- Hybridoma Center: The primary charter of this section was to provide monoclonal and polyclonal antibodies for research and for cloning purposes for the company.
- Miscellaneous:
  Helped establish Glaxo's U.S. R&D operation from ground zero. Helped R&D division grow from two employees and zero sq. ft to about 450 staff and more than 700,000 sq, ft. of lab space, helped establish and prioritize selected therapeutic areas for the U.S. operation. Helped in-licensing activities for the U.S. Corporation.

Head, Molecular Biology Department, Burroughs Wellcome Co., 1975-1986
The research emphasis in the Molecular Biology Department was centered on hormonal action, hormone receptors, and signal transduction mechanisms. Among the hormones investigated included, EGF, insulin, estrogen, progesterone, IGF-I, interferon, and opiate. Beside investigating enzymes like adenylate cyclase, phospholipase C, Phospholipase A2 and G-proteins, the Department also provided a general screening services to the Company for various receptors and selected enzymes.

Both functionally and administratively I was also responsible for The Wellcome Diagnostics Research. In addition to the administrative and scientific responsibilities of the Department, the following can be listed as major achievements of my own research activities.

· Established, organized, and conducted Therapeutic Advisory Panel.
· Purified and characterized estrogen receptors.
· Generated monoclonal antibodies to estrogen receptor (1977).

EXHIBIT 1

- Devised and patented commercially viable diagnostic assay for estrogen receptor.
- Established and patented a novel homogeneous enzyme immunoassay.
- Discovered and patented a non-absorbable antidiarrheal drug.
- Acted as Project Leader for preclinical work on the novel antidiarrheal drug.
- Cloned and expressed Cu-Zn Superoxide Dismutase (hSOD).
- Purified α interferon (1976)
- Actively evaluated external licensing candidates for both Burroughs Wellcome Co. and for Wellcome Diagnostics Ltd.

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June 2003

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Attorney Docket No.: 5051-451ip PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re: Yuehua Li, et al. Confirmation No.: 8515
Serial No.: 09/914,020 Group Art Unit: 1635
Filed: December 31, 2001 Examiner: Janet L. Epps-Ford
For: METHODS AND COMPOSITIONS FOR ALTERING MUCOUS SECRETION

Exhibit 2

EXHIBIT 2

Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 12290–12294, December 1995
Medical Sciences

Eosinophil recruitment into the respiratory epithelium following antigenic challenge in hyper-IgE mice is accompanied by interleukin 5-dependent bronchial hyperresponsiveness

(asthma/airway eosinophilia/mouse model)

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Communicated by K. Frank Austen, Harvard Medical School, Boston, MA, September 12, 1995

ABSTRACT **A murine model for antigen-induced bronchial hyperreactivity (BHR) and airway eosinophilia, two hallmarks of asthma, was developed using ovalbumin-immunized mice, which produce large amounts of IgE (named BP2, "Bons Producteurs 2," for High Line of Selection 2). A single intranasal ovalbumin challenge failed to modify the bronchial responses, despite the intense eosinophil recruitment into the bronchoalveolar lavage fluid and airways. When mice were challenged twice a day for 2 days or once a day for 10 days, BHR in response to i.v. 5-hydroxytryptamine or to inhaled methacholine was induced in BP2 mice but not in BALB/c mice. Histological examination showed that eosinophils reached the respiratory epithelium after multiple ovalbumin challenges in BP2 mice but remained in the bronchial submucosa in BALB/c mice. Total IgE titers in serum were augmented significantly with immunization in both strains, but much more so in BP2 mice. Interleukin 5 (IL-5) titers in serum and bronchoalveolar lavage fluid of BP2 mice were augmented by the antigenic provocation, and a specific anti-IL-5 neutralizing antibody suppressed altogether airway eosinophilia and BHR, indicating a participation of IL-5 in its development. Our results indicate that the recruitment of eosinophils to the airways alone does not induce BHR in mice and that the selective effect on BP2 mice is related to their increased IgE titers associated with antigen-driven eosinophil migration to the epithelium, following formation and secretion of IL-5.**

Bronchial hyperreactivity (BHR), a major characteristic of asthma, is associated with eosinophilic airway inflammation (1–3). By releasing their secretory products, eosinophils are claimed to damage bronchial epithelium and thus to induce BHR (3). This correlation has been demonstrated for guinea pigs (4), but until recently few studies have focused on the development of an eosinophil-dependent BHR model in mice. This may be very important, since such a model would allow exploration of the immunological mechanisms involved in BHR, particularly by taking advantage of the availability of mice strains and of specific tools. We demonstrated that Swiss, CBA, and hypereosinophilic interleukin 5 (IL-5) transgenic mice do not show BHR upon antigenic challenge, despite the marked eosinophil infiltration in airways (5), suggesting the need for factors other than eosinophil infiltration *per se*. The purpose of our study was to develop a model of BHR using a mice selection that produces high antibody titers in response to complex antigens [referred to as BP2, "Bons Producteurs 2"; see Biozzi *et al.* (6)] and that indeed showed very high IgE titers. Following repeated antigenic challenges, these animals became hypereosinophilic and hyperresponsive to nonspecific bronchoconstrictor substances. Our results suggest that BHR depends on an enhanced IL-5-driven production of eosinophils that locate in the respiratory mucosa, provided the microenvironment contains enough IgE antibodies.

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MATERIALS AND METHODS

Immunization and Provocation Procedures. Male BP2 (kindly provided by Denise Mouton, Institut Curie–Centre National de la Recherche Scientifique, Paris and by "Centre d'Elevage R. Janvier", BP5, 53940 Le Genest Saint-Isle, France) and BALB/c mice (Iffa-Credo and "Centre d'Elevage R. Janvier"), 8–10 weeks of age, were immunized s.c. twice at a 7-day interval with 0.4 ml of a solution of 250 μg of ovalbumin (OA) per ml (Immunobiologicals, Lisle, IL) mixed with 4 mg of $Al(OH)_3$ per ml (Merck). One week after the second injection, mice were challenged intranasally under light ether anesthesia with 10 μg of OA in 50 μl of 0.9% NaCl (saline) as indicated in *Results*. Control mice were challenged with the vehicle.

Evaluation of Bronchoconstriction. In a first procedure, mice were prepared as described (5), using the computerized pulmonary analyzer (Mumed PR800 system, UK) adapted to mice. To evaluate the effect of antigenic challenge on airway responsiveness, 5-hydroxytryptamine (5-HT; Sigma) was injected into the cannulated jugular vein at 10, 20, 40, 80, 160, and 320 μg/kg in a volume of 100 μl during 10 sec at 5-min intervals. The results were expressed as PD30, PD60, and PD90 (the amount of 5-HT needed to augment the bronchial resistance by 30%, 60%, and 90%). In a second procedure, unrestrained conscious mice were placed in a whole body plethysmographic chamber (Buxco Electronics, Sharon, CT) to analyze the respiratory waveforms. After a few minutes for stabilization, an aerosol of methacholine (3×10^{-2} M in the aerosolator; Aldrich) was delivered during 20 and 60 sec, at a 10-min interval. The airway resistance was expressed as Penh = [Te (expiratory time)/40% of Tr (relaxation time) − 1] × Pef (peak expiratory flow)/Pif (peak inspiratory flow) × 0.67, according to the recommendations of the manufacturer. To calculate the ΔPenh (difference between the basal and maximal value), the average of five maximal values was used.

Quantification of Serum IgE Levels. The total IgE titers were measured by ELISA according to Ledermann *et al.* (7).

Histological Evaluation. Lungs were prepared and processed for conventional histological studies. For optic microscopy, the microsections were stained with hematoxylin/eosin and for cyanide-resistant peroxidase (8). For electron micros-

Abbreviations: BALF, bronchoalveolar lavage fluid; BP2, "Bons Producteurs 2"; BHR, bronchial hyperreactivity; OA, ovalbumin; 5-HT, 5-hydroxytryptamine; PD30, PD60, and PD90, doses of 5-HT required to increase airway resistance by 30%, 60%, and 90%; IL, interleukin.

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copy, ultrathin sections were prepared with an LKB microtome, stained with uranyl acetate/lead citrate, and observed with a JEM-100CXII electron microscope.

For immunohistochemistry, 6-μm sections were prepared from OTC-inflated lungs and stained for murine $CD4^+$ and $CD8^+$ molecules using rat anti-murine CD4 antibody (L3/T4; Tebu, France) and rat anti-murine CD8 antibody (Ly-2; Tebu, France) and revealed with the alkaline phosphatase anti-alkaline phosphatase technique (9).

Statistical Analysis. The significance of differences was determined with the unpaired Student's *t* test.

RESULTS

Effect of Immunization and Antigen Challenge on the Cell Composition of the BALF. Total cell numbers (macrophages essentially) in the BALF of saline-challenged mice amounted to $0.85 \pm 0.06 \times 10^5$ cells per ml for BP2 and $1.23 \pm 0.1 \times 10^5$ cells per ml for BALB/c, eosinophils being absent. After a single antigen challenge, these numbers increased time-dependently, peaking at 96 hr (BP2, 3.95 ± 0.46 and $1.86 \pm 0.24 \times 10^5$ cells per ml; BALB/c, 2.70 ± 0.33 and $1.03 \pm 0.24 \times 10^5$ cells per ml, for total number and eosinophil number, respectively). These single-challenged mice failed to display BHR (see below) and, accordingly, more intense and/or prolonged stimuli were tested. Mice were challenged either twice a day for 2 days (group indicated as "2×2 days") or once a day for 10 days (group indicated as "1×10 days"). When the BALF was evaluated 24 hr after the last antigen challenge, the cell counts in BALF were increased above those following a single challenge. In particular, the 1×10 days group contained approximately 4.92 ± 0.7 (total cells) and 3.05 ± 0.54 (eosinophils) $\times 10^5$ cells per ml for BP2 and 6.02 ± 0.7 (total cells) and 2.99 ± 0.21 (eosinophils) $\times 10^5$ cells per ml for BALB/c, respectively. Lymphocytes were absent in controls but were present in both groups of multichallenged mice, neutrophil counts being unaffected.

Effect of Antigen Challenge on Bronchial Responsiveness to i.v. 5-HT in Anesthetized Mice. Single-challenged BP2 mice showed no significant augmentation of bronchial responses to 5-HT (Fig. 1*A* for 24 hr; other time points not shown). However, BHR was induced following repeated challenge with antigen (Fig. 1*A*). By contrast, BHR was not uncovered in BALB/c mice (Fig. 1*B*), even though the eosinophil numbers were comparable to those of BP2. BHR was also absent in response to methacholine in BALB/c mice, no matter what the provocation protocol (data not shown). No statistical correlation (by linear regression analysis) was found between eosinophil numbers in BALF and the intensity of bronchoconstriction in all OA-multichallenged BP2 mice, whether from the groups 2×2 days and 1×10 days.

Effect of Immunization on the IgE Serum Titers of BALB/c and BP2 Mice. Nonimmunized BP2 mice contained 3.35 ± 2.57 μg of total IgE per ml and BALB/c contained 0.18 ± 0.05 μg/ml. Immunization induced a significant augmentation of those titers in both strains, the total amount reaching 24.96 ± 5.84 μg/ml and 0.68 ± 0.11 μg/ml, in BP2 ($P < 0.05$) and BALB/c ($P < 0.05$) mice, respectively.

Effect of Antigen Challenge on Bronchial Reactivity to Inhaled Methacholine in Nonanesthetized Mice. Our inability to induce BHR in BALB/c mice might be attributed to failure of 5-HT to reach the appropriate sites in the airways, since it was injected i.v. A group of immunized and nonanesthetized mice challenged using the protocol 2×2 days was thus evaluated. OA-challenged BP2 mice showed a significant augmentation of airway resistance in response to methacholine as compared to control animals, whereas, once again, no BHR was observed in BALB/c mice (Fig. 2).

Effect of Immunization and Antigen Challenge on the Lung Histology. No histological abnormalities were observed in lungs from control mice of both strains (Fig. 3*a*). In antigen-challenged mice, granulocytes (mainly eosinophils and a few neutrophils), lymphocytes, plasma cells, and some macrophages accumulated in the peribronchial and perivascular tissues. A moderate submucosal eosinophil infiltration was observed in BP2 and BALB/c mice (30 ± 6 and 29 ± 4 cells per mm, respectively) challenged once and killed after 24 hr, with practically no eosinophils present in the epithelium. More eosinophils were found in the submucosa of 2×2 days mice (120 ± 7 cells per mm for BP2 and 46 ± 10 cells per mm for BALB/c). Under those conditions, the epithelium of BALB/c mice was still eosinophil-free, but eosinophils clearly infiltrated that of BP2 mice (23 ± 7 cells per mm) (Fig. 3*b*). This epithelial and submucosal eosinophil infiltration in BP2 mice was better appreciated in sections stained for eosinophil peroxidase. Finally, plasma cells were more abundant in the submucosa of BP2 mice (Fig. 3*d*) as compared to BALB/c mice.

Both mice strains showed a multifocal perivascular and peribronchial eosinophilic alveolitis with eosinophils, macrophages, and few giant cells, even after a single antigenic chal-

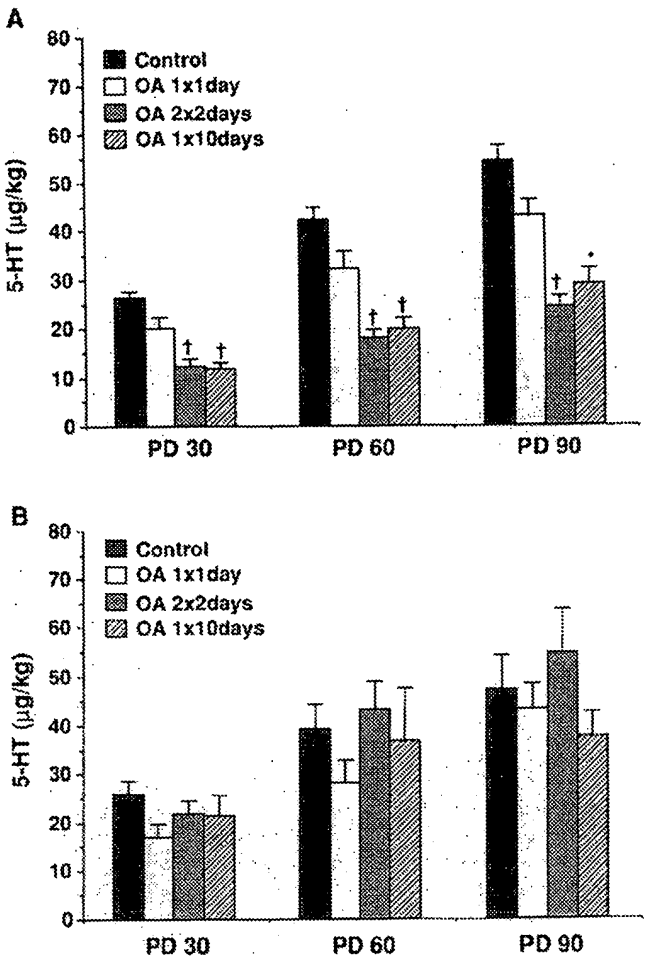

FIG. 1. Airway responsiveness in immunized and antigen-challenged BP2 (*A*) and BALB/c (*B*) mice following different protocols for antigen challenge. In the protocol 1×1 day, 10 μg of OA in 50 μl of 0.9% NaCl was instilled at ≈9 a.m. and BHR was evaluated after 24 hr (see text). In the protocol 2×2 days, OA as above was instilled at 9 a.m. and at 5 p.m.—i.e., at an 8-hr time interval, for 2 days. On the 3rd day, BHR was evaluated. When the protocol 1×10 days was used, OA as above was instilled at 9 a.m. for 10 days. On the 11th day, BHR was evaluated. Controls were saline-challenged mice of each group, which were pooled. Only the BP2 mice challenged 4 (▩) or 10 (▨) times showed a significant augmentation (†, $P < 0.001$, or *, $P < 0.005$, respectively) of the intensity of bronchoconstriction, compared to control mice. Each value represents the mean ± SEM of 5–14 mice.

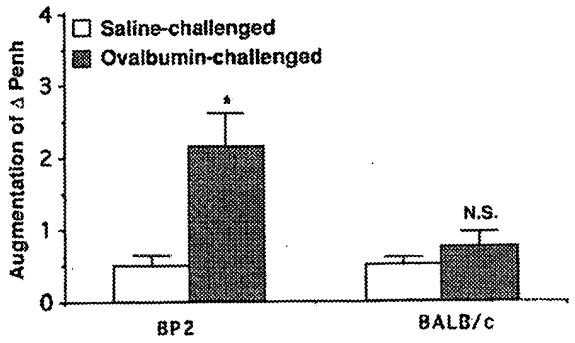

FIG. 2. Airway responsiveness in immunized and antigen-challenged BP2 and BALB/c mice in response to inhaled methacholine (3×10^{-2} M) in the absence of anesthesia. Mice were challenged with OA (■) twice daily for 2 days (protocol 2×2 days) and tested 24 hr after the last challenge. Methacholine was inhaled for 20 and 60 sec at a 10-min interval. The results for 20 sec are shown; similar results were obtained for 60 sec. The results are expressed in ΔPenh (see text). Each value represents the mean ± SEM of 6–10 mice. Significant differences compared with control (□) are indicated: *, $P < 0.05$ and nonsignificance is expressed as N.S.

lenge. Under conditions where lungs from immunized saline-challenged mice contained no T lymphocytes in the bronchial wall, $CD4^+$ and $CD8^+$ T lymphocytes were identified in two BP2 mice challenged with the protocol 2×2 days (30 CD4 and 19 CD8 T cells per mm of bronchial wall).

Electron Microscopy. Ultrastructural examination confirmed that the majority of the granulocytes observed in the bronchial walls were eosinophils. Eosinophil secondary granules in the epithelium and in the submucosa were of two types, one with a central electron-dense core surrounded by a lighter area and a second showing the reverse—i.e., an electron-dense periphery and a central electron-light core. The morphology of the granules was identical in both strains, with no evidence of degranulation or for differences in number of granules. Thus, an average of 22.7 ± 1.7 and 19.44 ± 3 granules per eosinophil section of BP2 mice was scored in the submucosa ($n = 18$ cells) and in the epithelium ($n = 9$ cells), respectively. Similar values were obtained for eosinophils of BALB/c mice counted in the submucosa.

Interference of the Anti-IL-5 Antibody TRFK-5 with Eosinophilia and BHR. Approximately 150 pg of IL-5 per ml was found in the serum and 300 pg/ml in the BALF of BP2 mice 1–6 hr after the fourth antigenic provocation (protocol 2×2 days). This led us to evaluate the anti-IL-5 antibody TRFK-5. In these experiments, OA challenges augmented the total cell numbers in the BALF of IgG isotype-treated mice to 6.3 ± 0.8 × 10^5 cells per ml ($P < 0.001$), a number comparable to that of saline-treated animals (Fig. 4*A*). This increment was accounted for by a marked increase in the proportion of eosinophils (57.5% ± 2.5% of total cell numbers). TRFK-5, injected i.v. at 0.5 mg per mouse 1 hr before each OA challenge, reduced significantly the total BALF cell counts, which were accounted for essentially by decreased eosinophil numbers (isotype, 3.54 ± 0.38 × 10^5 cells per ml; TRFK-5, 0.58 ± 0.25 × 10^5 cells per ml; $P < 0.05$) (Fig. 4*A*). Histological observations confirmed that the administration of TRFK-5 inhibited the epithelial (from 6.8 to 0.7 cells per mm) and submucosal (from 55.7 to 14.2 cells per mm) eosinophil infiltration in two mice.

As expected, OA challenges in saline or IgG isotype-treated mice resulted in BHR (Fig. 4*B*). TRFK-5 inhibited the antigen-induced BHR for 5-HT in anesthetized (Fig. 4*B*) and for methacholine in nonanesthetized antigen-challenged mice (Fig. 4*C*).

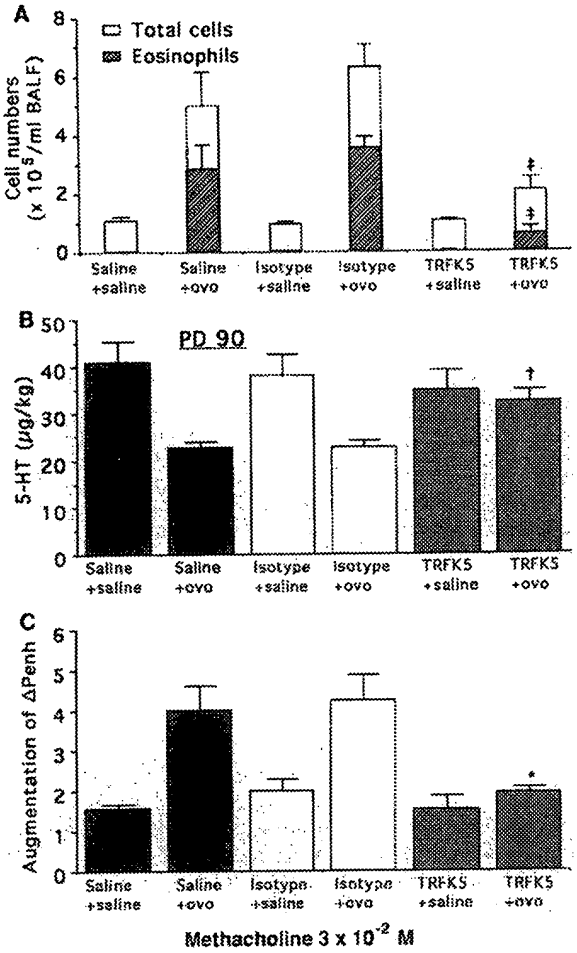

FIG. 4. Effect of the anti-IL5 antibody TRFK-5 on eosinophil recruitment into the BALF (*A*) and on BHR (*B* and *C*) in immunized BP2 mice. The protocol 2×2 days was used. Purified IL-5 antibody from TRFK-5 cells was injected i.v. at 0.5 mg per mouse to immunized BP2 mice, 1 hr before each of the four OA (ovo) challenges. The same volume of control antibody (rat IgG) was injected into control mice. Bronchoalveolar lavage and BHR were evaluated 24 hr after the last antigen challenge. BHR with 5-HT was expressed as PD90 (see text) in anesthetized mice and BHR with methacholine was expressed as ΔPenh (see text) in nonanesthetized mice. Each value represents the mean ± SEM of 4–12 mice. Significant differences compared with control isotype (challenged with OA) are indicated: *, $P < 0.05$; †, $P < 0.005$; ‡, $P < 0.001$.

DISCUSSION

Eosinophils are recruited to the airways of asthmatic patients and are considered important effector cells in BHR, a hallmark of asthma (10). Although BHR is reported to correlate with the number of eosinophils in lungs staining positively with a monoclonal antibody that recognizes secreted eosinophil cationic protein (10–13), there is no formal evidence for a causal relationship between eosinophil recruitment *per se* and BHR in humans (14, 15), sheep (16), and guinea pigs (4). Few studies have addressed the correlation between eosinophil recruitment to the airways and BHR in mice, possibly because the evaluation of their pulmonary functions was considered difficult or unreliable. Nevertheless, murine models should be invaluable for dissecting the mechanisms of BHR, since murine basic immunology is well defined. This led us to search for and to identify mice that displayed intense anaphylactic responses, in addition to antigen-induced hypereosinophilia. We took advantage of the availability of high-responder mice (detailed in ref. 6) selected by bidirectional breeding for antibody responsiveness to sheep erythrocytes in terms of agglutinin titers, which have been shown to be homozygous for the character considered in F_{14}–F_{17} generations (6, 17). These mice developed BHR, which was detected by two methods. In

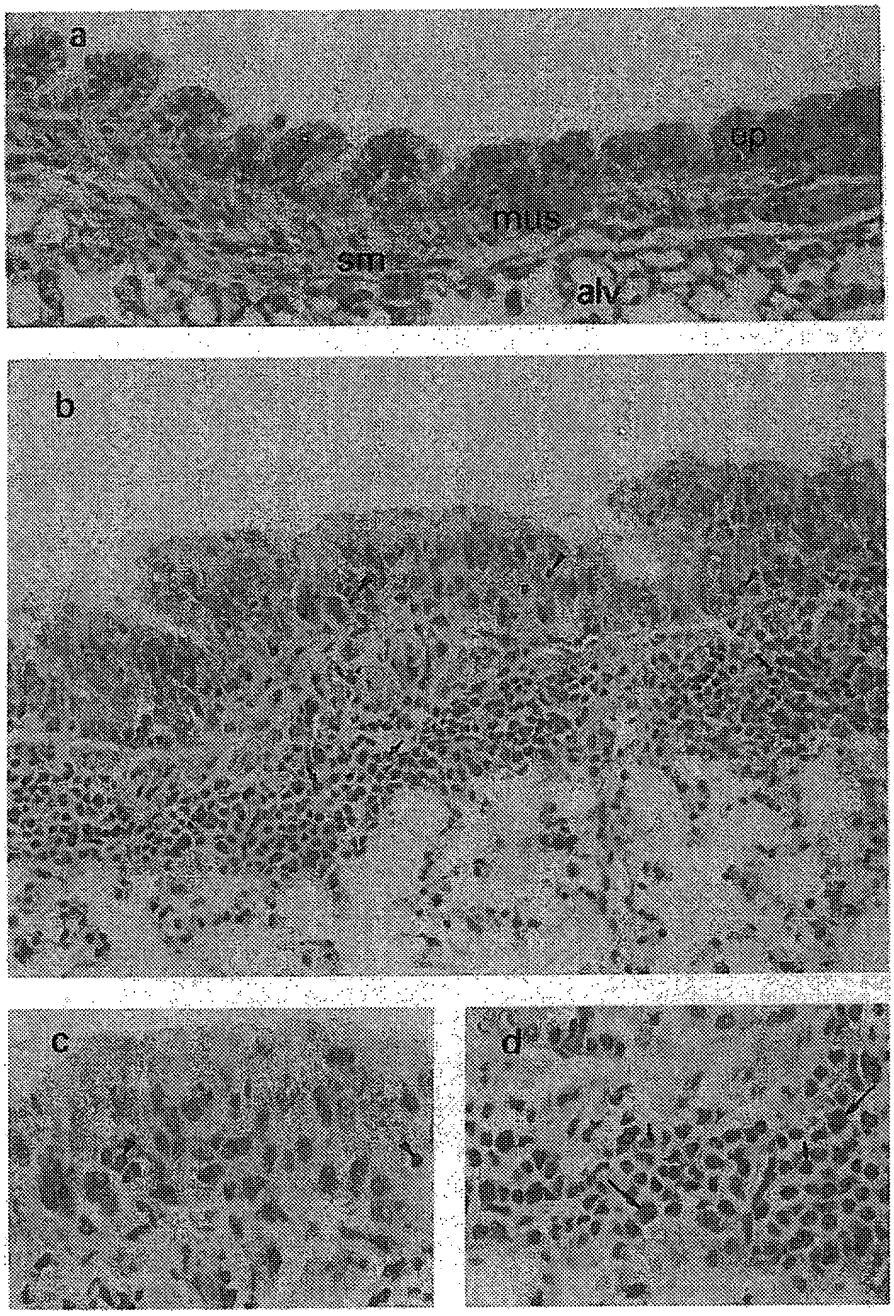

FIG. 3. (*a*) Lobar bronchus from BP2 mice immunized with OA and challenged with saline. Control BALB/c mice showed the same morphology as BP2 mice. Note the absence of inflammatory cell infiltration. ep, Epithelium; mus, bronchial muscle; sm, submucosa; alv, alveolus. (×70.) (*b*) Lobar bronchus from BP2 mice challenged with OA according to protocol 2×2 days. A marked eosinophil infiltration of the submucosa (three short arrows) and of the mucosa (three arrowheads) is noted. (×70.) (*c*) Higher magnification of the section in *b* showing the mucosa containing numerous infiltrating eosinophils (two arrowheads). (×280.) (*d*) Higher magnification of the mucosa and submucosa showing the dominant infiltrating eosinophils (short arrows) but also a number of plasma cells (long arrows). (×280.) All sections were stained with hematoxylin/eosin.

a first one, 5-HT was administered i.v. to anesthetized mice (5, 18), and in the second, methacholine was administered by aerosol to awake animals (19). This is important since bronchoconstrictor responses may differ according to the route of administration of the agonists, which determines the first line cell species that is encountered. Furthermore, since eosinophils activated *in situ* might induce BHR by affecting the respiratory epithelium (3), it seemed logical to deliver the bronchoconstrictor agent directly into the airways. Both methods provided similar results.

BP2 mice differed from BALB/c mice in at least four different ways. (*i*) A first and essential difference is that only BP2 mice displayed BHR following multiple antigenic challenges. (*ii*) They showed very high levels of serum IgE, which doubled after immunization, whereas BALB/c mice had considerably lower amounts, which, as expected, also augmented upon immunization. (*iii*) BP2 mice underwent an intense anaphylactic bronchoconstriction when challenged with i.v. OA, whereas BALB/c were unresponsive (not shown). (*iv*) Eosinophils were identified in the epithelium of BP2 mice after antigen challenges, being absent in BALB/c mice. In previous studies, we also failed to identify eosinophils in the epithelium of antigen-challenged mice from other strains, including IL-5 transgenic animals, even though the BALF and the submucosa were heavily enriched with eosinophils (5). The basic difference between BP2 and other mice in terms of eosinophils thus appears to relate to their topographic distribution and not to absolute counts. It was tempting to speculate that eosinophils stimulated at the vicinity of the targeted respiratory epithelium, in a hyper-IgE environment, are likely to become activated, particularly after repeated antigenic challenges. Nevertheless, the secondary granules of the eosinophils found in the epithelium and in the submucosa did not show morphological evidence of degranulation, according to published criteria (20, 21). It is thus unlikely that eosinophils participate in the induction of BHR in BP2 mice, via the secretion of cationic proteins such as "Major Basic Protein" as in guinea pigs (4), and it also remains to be shown whether an augmented

production of lipid mediators may occur. Eosinophils from immunized and aerosol-challenged guinea pigs are indeed primed to produce larger amounts of leukotriene C4 than control animals (22). On the other hand, the respiratory epithelium might express adhesion proteins in the hyper-IgE environment in BP2 mice, leading to the recruitment and activation of eosinophils. Indeed, we demonstrated that an antibody against very late activation antigen 4 suppresses the antigen-induced airway eosinophil recruitment and *in situ* activation and the associated BHR in guinea pigs (23).

Our results contrast to those of Renz *et al.* (18), who showed that BALB/c mice immunized by inhaled OA for a long time interval display BHR in response to i.v. methacholine. This may result from different protocols, particularly since no eosinophils or other inflammatory cells were found in the airways of these mice at the time of BHR but might have occurred before. BHR persists in BP2 mice for at least 16 days, a time when eosinophils are absent (not shown).

In immunized BP2 mice, the IgE titers were very elevated and the number of plasma cells identified in the bronchial submucosa was increased, two possibly related events. Increased IgE titers may be involved in the induction of BHR, particularly since an anti-IgE antibody inhibits altogether airway eosinophilia and BHR in our model (data not shown). IgE production is dependent on IL-4 (24), a Th2 lymphocyte-derived cytokine. IL-4 is also involved in eosinophil recruitment to the airways upon antigen challenge (25), but it is clear that IL-5 is responsible for eosinophil recruitment to the airways in the mice strains studied and for BHR, when applicable. Indeed, IL-5 was present in the BALF and serum of the BP2 mice after antigen challenge and the anti-IL-5 antibody TRFK-5 suppressed altogether eosinophilia in airways and tissues and BHR. In allergen-challenged guinea pigs, anti-IL-5 antibody also suppressed airway eosinophilia (26) and BHR (27, 28). $CD4^+$ T lymphocytes that migrate into the lungs after antigen challenge (29) are responsible for the local production of IL-4 and IL-5, which are important in B- and T-lymphocyte development and function and in eosinophil activation and differentiation (30). In $CD4^+$ deficient or immune-depleted mice, BALF eosinophilia and airway inflammation in response to antigen provocation were absent (25, 31) and BHR was reduced (25). In confirmation, we identified $CD4^+$ T cells in the bronchial wall after antigen challenge in BP2 mice, suggesting that these cells participate in the induction of airway eosinophilia, and accordingly of BHR, by releasing cytokines.

As a conclusion, repeated intranasal antigenic challenges induce eosinophil recruitment to the lungs and to the BALF in BALB/c and in BP2 mice, but BHR develops selectively in the latter. Since eosinophil recruitment to the lungs and BHR were inhibited by *in vivo* IL-5 neutralization, eosinophils are clearly necessary, but not sufficient, for BHR. Our finding that eosinophils locate in the respiratory epithelium only in hyper-IgE BP2 mice, which become hyperresponsive, suggests that BHR involves the interaction between eosinophils recruited and/or primed by IL-5 and a component of the epithelium in the presence of an altered microenvironment, including high IgE titers. Interference with one of the sequential events involved—i.e., excessive eosinophil production or recruitment, the selective expression of adhesion proteins on the endothelial or epithelial surfaces, the production or the effects of IgE, or finally the production or effects of eosinophil-borne mediators—should be sufficient to suppress BHR.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re: Yuehua Li, et al. Confirmation No.: 8515
Serial No.: 09/914,020 Group Art Unit: 1635
Filed: December 31, 2001 Examiner: Janet L. Epps-Ford
For: METHODS AND COMPOSITIONS FOR ALTERING MUCOUS SECRETION

Exhibit 3

EXHIBIT 3

Mucin Secretion in a Murine Model of Asthma

Peptide	% Mucin Secretion	% Inhibition of Mucin Secretion Relative to OVA Control
Untreated Saline Control	8	92
OVA Control	100	0
BIO-124R (RNS)	116	-16
BIO-124 (MANS)	24	76
BIO-120	23	77
BIO-116	32	68
BIO-112	23	77
BIO-110	34	66